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Characterization of Gla-Containing Proteins From Calcified Human Atherosclerotic Plaques

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INTRODUCTION

Besides in the liver, vitamin K-dependent carboxylase also occurs in many non-hepatic tissues including the vessel wall [1]. The Gla-containing proteins produced by the hepatic enzyme are excreted into the blood stream where at least six of them are involved in the blood coagulation process. They all belong to the circulating Gla-proteins. A second class of Gla-proteins comprises those found in calcified tissues such as bone, dentin, renal stones and hardened atherosclerotic plaques [2,3]. In this paper we will focus our attention to the plaque proteins. Obviously they might originate either from the blood stream from which they could have been adsorbed or, alternatively, they might be produced by vessel wall carboxylase. Before information about their origin may be obtained, a thorough characterization of these proteins seems to be warranted.

The information obtained from the literature is confusing. In 1979 a Gla-containing protein was discovered in hardened atheromatous plaques and because of its calcium-binding properties it was designated as atherocalcin [2]. Later on it was reported by the same group that atherocalcin was merely a complex between osteocalcin and albumin [3] and that this complex accounted for only a small part of the total amount of Gla-residues in EDTA-extracts of calcified plaques. The nature of the remaining proteins has remained obscure up till now. We have tried to characterize more completely the protein constituents of calcified plates in human aortae and the results of our investigations are presented in this paper.

MATERIALS AND METHODS

Chemicals. Benzamidine hydrochloride was obtained from Janssen Pharmaceutica (Belgium), trasylol from Bayer (F.R.G.) and human serum albumin from Nordic Laboratories (The Netherlands). Soybean trypsin inhibitor and guanidine hydrochloride (guanidine/HCl) were purchased from Sigma (U.S.A.) $^{45}CaCl_2$ (1.8 Ci/mmol) was obtained from Amersham International (U.K.) and Atomlight from New England Nuclear (F.R.G.) CNBr-activated Sepharose and QAE-Sephadex were purchased from Pharmacia (Sweden) and NH_4HCO_3 from Baker Chemicals (The Netherlands). All other chemicals were obtained from Merck (F.R.G.). Spectrapor 3 dialysis tubing (molecular weight cutt of: 3,500 D) was from Spectrum Industries (U.S.A.) and was used for all dialyses.

Antibodies. Osteocalcin was purified as described earlier [4]. Antibodies against this protein were raised in rabbits and purified from the crude immune sera by immunoaffinity chromatography. Antibodies against purified human prothrombin and factor X were purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (The Netherlands), antibodies against human serum albumin were from Nordic Laboratories (The Netherlands) and antibodies against human protein C were a kind gift from Dr. R. Bertina (Leiden, The Netherlands).

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Tissue preparation. Human atherosclerotic aortae (n=50) were obtained at autopsy between 5 and 24 h after death and stored at -60 °C until use. The aortae were dissected free of surrounding tissue and fat and rinsed in buffered saline to remove contaminating blood. Areas of discrete, hard calcification were excised and pooled. Subsequently a liquid nitrogen milled powder was prepared from these tissue pools and 50 g of powder was extracted for 8 days at 4 °C in a buffer containing 1.0 M EDTA, 0.2 M KCl, 4.0 M guanidine/HCl (pH 8.0) in the presence of the following protease inhibitors: benzamidin (0.01 M), trasylol (100,000 KIE per litre) and soybean trypsin inhibitor(0.02%, w/v). The extraction buffer was renewed after 4 days. Insoluble material was removed by centrifugation at 10,000 x g for 15 min at 4 °C and the proteins extracted were diluted 20-fold with distilled water followed by a batchwise adsorption onto QAE-Sephadex in 0.15 M NaCl, 0.05 M Tris/HCl (pH 8.0) using 10 ml of slurry per l diluted extract. Subsequently the adsorbed proteins were eluted with 1.0 M NaCl, 0.05 M Tris/HCl (pH 8.0) and the eluate was dialyzed extensively against distilled water to remove EDTA and other salts. After lyophilization and another extensive dialysis against 0.05 M NH_4HCO_3, the protein fraction obtained was centrifuged at 2,000 x g for 10 min at 4 °C. The resulting supernatant was designated as the calcium plate extract.

Precipitation-inhibition studies. The protein solutions to be tested were dialyzed against 0.15 M NaCl, 0.05 M Tris/HCl (pH 7.4) prior to use. Non-labeled $CaCl_2$ was supplemented with 20,000 dpm of the tracer ($^{45}CaCl_2$) and the proteins to be tested. The optimal reaction conditions for studying the calcium phosphate precipitation were: 5 mM $CaCl_2$, 150 mM NaCl and 5 mM sodium phosphate, pH 7.4. The mixtures were incubated in Eppendorf tubes for 2 h at 37 °C and the precipitate formed (if any) was spun down at 2,000 x g. Subsequently 0.5 ml from the supernatant was taken and counted in a Beckman LS 1801 liquid scintillation counter using Atomlight as a scintillation liquid. For the precipitation studies with calcium carbonate we used a freshly prepared solution containing 250 mM sodium bicarbonate, the pH of which had been adjusted to 7.4 by slowly adding 1 M HCl. The reaction mixtures contained 5 mM $CaCl_2$, 150 mM NaCl, 125 mM $NaHCO_3$ and proteins as indicated. The extent to which a certain protein was able to inhibit the precipitation of calcium salts was quantitated as follows. The radiolabel which had remained in solution in the absence of any protein was regarded as a blank value and was subtracted from the data obtained in the presence of the protein. Subsequently the difference between these two figures was expressed as a percentage of the total amount of label added to the reaction mixture.

Various assays. The composition of the mineral deposits of human atherosclerotic aortae was determined by infra-red spectroscopy analysis and by stone analysis (ready prepared kit from Temmler Diagnostika (FRG)). Protein concentrations were measured according to Sedmak and Grossberg [5] and Gla and Glu residues were determined after alkaline hydrolysis of the proteins as in [6]. Decarboxylation of Gla-containing proteins was accomplished as described in [7]. The presence of osteocalcin was determined with a radioimmunoassay (Immuno Nuclear Company). High performance liquid chromatography (HPLC) was carried out using a Bio-Sil TSK-250 size exclusion column (Bio-Rad, 600x21.5 mm). Polyacrylamide gel electrophoresis in the presence of SDS was performed with ready prepared Phast Gel gradient 10-15 gels with a continuous gradient from 10 to 15 % polyacrylaimde in combination with Phast Gel SDS buffer strips. The gels were stained with Coomassie Brilliant Blue and destained following the recommendations of the manufacturer. The Phast Gel electrophoresis system as well as the ready prepared polyacrylamide Phast Gels were products of Pharmacia (Sweden).

RESULTS

Sediment analysis of a powder of 50 g of calcified plaques demonstrated that the mineral phase mainly consisted of calcium phosphate and calcium

carbonate. Since the procedure used to solubilize the mineralized material includes a dialysis step of 1 week it may be expected that low molecular weight material (e.g. free or peptide-bound Gla) had been removed from the organic phase. Yet substantial amounts of Gla were detected in alkaline hydrolysates of the calcium plate extract (data not shown).

TABLE I. Characterization of the calcium plate extract.

Sepharose-bound antibodies	Gla (ng/ml)	Protein (mg/ml)	Gla/Protein (ng/mg)
a. None	315.0	0.41	768.3
b. Murine IgG	302.0	0.40	755.0
c. Anti-prothrombin	292.5	0.39	750.2
d. Anti-factor X	292.0	0.39	748.7
e. Anti-protein C	290.0	0.39	743.6
f. Anti-osteocalcin	254.4	0.40	636.0
g. Anti-albumin	273.6	0.38	729.0
h. f + g	221.2	0.39	567.2

Samples containing 0.2 ml calcium plate extract, 0.1 ml of Sepharose slurry and 0.1 ml of buffer 0.1 ml NaCl, 0.05 M Tris/HCl (pH 7.4) were rotated end over end overnight at 4 ^{o}C. Subsequently the tubes were centrifuged for 5 minutes at 2000 x g and aliquots (0.2 ml) from the supernatants were taken for protein and Gla determination. Immobilized murine IgG was used as a control to exclude non-specific binding to the Sepharose. It was verified in all cases that increasing the amount of Sepharose-bound antibodies did not affect the data. In the experiment described in line a buffer was added in stead of Sepharose. The results are the mean values of duplicate measurements.

We have investigated if the Gla-proteins in the calcium plate extract were related to one of the most abundant Gla-proteins in plasma (prothrombin, factor X and protein C) or to osteocalcin and/or albumin. These experiments were perfomed by incubating the extract with immobilized immuno-purified antibodies against each of the various proteins. The decrease of the total amount of Gla in the supernatant fraction was taken as a measure for the amount of antigen removed. The results of these experiments are summarized in table I and it is obvious that none of the (anti-)coagulation factors could be detected in the plate extract. Only the antibodies against osteocalcin and -to a lesser degree- albumin were able to bind part of the Gla-proteins, but even the combination of the two antibodies resulted in a removal of only one third of the protein-bound Gla residues. The presence of osteocalcin in the calcium plate extract was confirmed by radioimmuno assay, which showed that the preparation contained 210 ng osteocalcin per mg of protein. This is somewhat less than the amount calculated from table I, but it should be kept in mind that if osteocalcin occurs as a complex with albumin the data obtained by RIA will probably underestimate the actual osteocalcin concentration.

We have also investigated to what extent the extracted plaque proteins affected the in vitro precipitation of calcium phosphate and of calcium carbonate from a supersaturated solution. These two salts are the main constituents of the inorganic phase of the plaques. It resulted that at a physiological pH (7.4) the plate extract strongly inhibits the precipitation of calcium carbonate (fig. 1A) and calcium phosphate (data not shown). Human serum albumin (lacking Gla residues) had no effect, even at concentrations up to 50 µg/ml. The role of Gla-residues in the precipitation-inhibitory activity was demonstrated in an experiment in which the

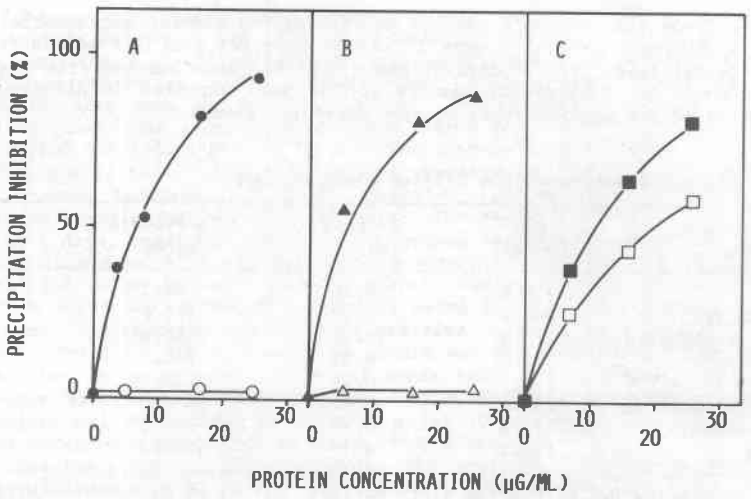

Fig. 1. Effect of various proteins on the precipitation of calcium carbonate. The precipitation-inhibition activity of the calcium plate extract was measured before (A) and after (B) thermal decarboxylation and after removal of osteocalcin (C).
The incubations were performed for 2h at 37 °C as described in the Material and Methods section. Explanation of symbols: ●-●, calcium plate extract; ○-○, human serum albumin; ▲-▲, d-calcium plate extract pH 8; △-△, d-calcium plate extract pH 2; ■-■, calcium plate extract after treatment with anti-osteocalcin; □-□, calcium plate extract after treatment with anti-osteocalcin and anti-albumin.

plate extract was dialyzed against distilled water and divided into two parts, one of which was brought to pH 2.0 and the other one to pH 8.0. After lyophilyzation both preparations were subjected to the conditions of thermal decarboxylation (24 h at 110 °C in vacuo) and redissolved in Tris-buffered saline (pH 7.4). The pH was checked at this stage and adjusted, if required. The acid-treated preparation did not contain protein-bound Gla-residues any more, whereas Gla in the other sample had remained unchanged. As shown in fig. 1B, only the Gla-containing sample had retained its precipitation-inhibitory activity. Since also osteocalcin has been reported to inhibit the precipitation of insoluble calcium salts [8] the calcium plate extract was depleted of osteocalcin and of albumin/osteocalcin complexes (if any) by incubating the sample with an excess of Sepharose-bound antibodies against osteocalcin and albumin. As shown in fig. 1C, also the osteocalcin-depleted plate extract strongly inhibited the precipitation of calcium carbonate. From these data we concluded that the calcium plate extract must contain a hitherto unidentified Gla-protein and we have decided to purify the latter.

The crude plaque proteins were fractionated on a size exclusion column using high performance liquid chromatography. The varous peak fractions were pooled as indicated in fig. 2 and analyzed for their Gla content. Gla was mainly present in peak nr. 5 and rechromatography of this sample showed one single peak eluting at a position corresponding to a mass of approximately 20 kD. Also analysis on polyacrylamide gels in SDS showed a single protein band with an apparent mass of 20 kD, which did not change under reducing conditions. The preparation thus obtained still strongly inhibited the in vitro precipitation of calcium salts. Its biochemical characterization is now in full progress and will shortly be reported elsewhere.

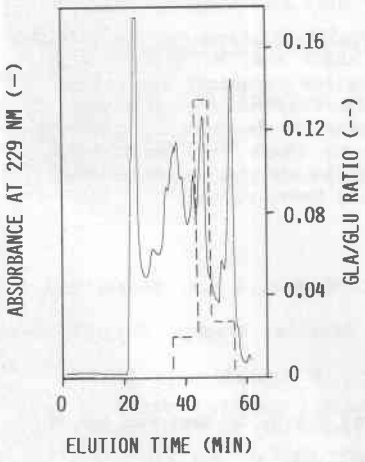

Fig. 2. Fractionation of calcium plate extract on a Bio-Sil TSK-250 column. The flow rate was adjusted to 240 ml/h and the absorbance of the effluent was monitored at 229 nm (solid line). The various peak fractions were pooled and analyzed for their Gla content (dashed line).

DISCUSSION

Amongst the Gla-containing proteins bound to calcified atherosclerotic plaques we have been unable to detect prothrombin, factor X or protein C. Since these three proteins are the most abundant Gla-proteins in plasma we assume that adsorption of the proteins involved in blood coagulation to the calcifying vessel wall hardly occurs. Osteocalcin, on the other hand, is found in plasma in extremely low concentrations, but substantial amounts could be identified in the calcium plate extract. This observation might be explained by its low mass (6 kD), which might facilitate its penetration into the tissue surrounding the plaque, or by its high affinity for hydroxylapatite and other calcium salts. The upper limit of the sum of free osteocalcin and that complexed to albumin was determined to be 30% of the total amount of Gla-proteins.

After removal of all osteocalcin from the sample, the remaining preparation still strongly inhibited the in vitro precipitation of calcium salts and its was made plausible that one or more Gla-proteins were responsible for this activity. Fractionation of the sample by HPLC lead to the purification of a Gla-containing protein with an apparent mass of 20 kD. Since atherocalcin is a trivial name, we will designate the 20 kD protein we found as plaque Gla-protein.

The pathophysiological importance of the plaque Gla-protein remains a matter of speculation. Because of its strong inhibition of calcium salt precipitation in vitro, it might play a role in preventing the calcification of atheromatous plaques. If for some reason (e.g. a decreased production of plaque Gla-protein) calcification of the vessel wall does occur, the protein is expected to bind to the calcified material thus retarding the calcification process without preventing it. This would explain why it can be isolated from the calcified plates.

Antibodies against the purified plaque Gla-protein have been raised and were successfully used to develop an enzyme linked immunosorbent assay (ELISA). Immuno-purified antibodies will be used for the cytochemical detection and localization of plaque Gla-protein in vessel wall, whereas the ELISA will be used to measure its concentration (if any) in plasma from normal subjects and from atherosclerotic patients. These investigations are in current progress in our laboratory.

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